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Quantification of the co-mutagenic β-carbolines, norharman and harman, in cigarette smoke condensates and cooked foods

Yukari Totsuka[a], Hirofumi Ushiyama[b], Junko Ishihara[a], Rashmi Sinha[c], Sumio Goto[d], Takashi Sugimura[a], Keiji Wakabayashi[a,*]

[a]*Cancer Prevention Division, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan*
[b]*Division of Food Hygiene, The Tokyo Metropolitan Research Laboratory of Public Health, 24-1 Hyakunincho 3-chome, Shinjuku-ku, Tokyo 169-0073, Japan*
[c]*Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD 20892, USA*
[d]*Department of Community Environmental Sciences, National Institute of Public Health, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8638, Japan*

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Abstract

Co-mutagenic β-carbolines, such as norharman and harman, were quantified in mainstream and sidestream smoke condensates of six Japanese brands of cigarettes, and also in 13 kinds of cooked foods, using a combination of blue cotton treatment and HPLC. Norharman and harman were detected in all the cigarette smoke condensate samples. Their levels in the mainstream smoke case were 900–4240 ng per cigarette for norharman, and 360–2240 ng for harman, and in sidestream smoke, 4130–8990 ng for norharman and 2100–3000 ng for harman. These β-carbolines were also found to be present in all the cooked food samples, at levels of 2.39–795 ng for norharman and 0.62–377 ng for harman per gram of cooked food. The observed concentrations are much higher than those found for mutagenic and carcinogenic heterocyclic amines (HCAs), suggesting that humans are exposed to norharman and harman in daily life to a larger extent than to HCAs.

Keywords: Norharman; Harman; Cigarette smoke; Cooked food

1. Introduction

The β-carboline compound, norharman (9*H*-pyrido[3,4-*b*]indole), is itself not mutagenic to *Salmonella typhimurium* strains, either with or without an S9 mix, but shows mutagenicity in TA98 and YG1024 with an S9 mix in the presence of non-mutagenic aromatic amines including aniline and *o*-toluidine [1–3]. It has therefore been termed a 'co-mutagen'. Moreover, DNA adduct formation by norharman with aromatic amines has been demonstrated to be related to the co-mutagenic action of norharman in *S. typhimurium* TA98 [4]. Another β-carboline compound, harman (1-methyl-9*H*-pyrido[3,4-*b*]indole), has also been reported to show co-mutagenic activity, although its activity is less than that of norharman [3].

By HPLC purification, we have isolated two mutagenic compounds, produced by the reaction of norharman with aniline, one showing mutagenicity with, and the other without, an S9 mix. The former was determined to be a coupled compound of

* Corresponding author. Tel.: +81-3-3542-2511; fax.: +81-3-3543-9305.
E-mail address: kwakabay@gan2.ncc.go.jp (K. Wakabayashi)

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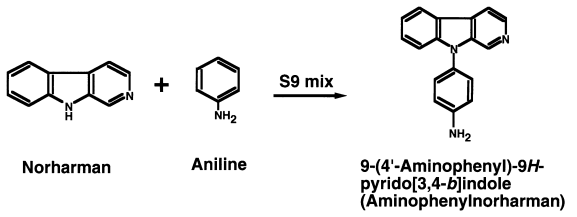

Fig. 1. Formation of mutagenic 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole (aminophenylnorharman) by a reaction of norharman with aniline in the presence of an S9 mix

norharman and aniline, 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole (aminophenylnorharman), inducing 1 783 000 revertants in YG1024 per μg with an S9 mix. The latter was hydroxyaminophenylnorharman. The same DNA adducts were formed when aminophenylnorharman alone or a mixture of norharman with aniline was incubated with an S9 mix and YG1024. Thus, the appearance of mutagenicity in this case was suggested to be due to the formation of the coupled compound, as shown in Fig. 1 [5]. Aminophenylnorharman is converted to the hydroxyamino derivative which further activated to yield esters, and finally the ultimate forms react with DNA bases to induce mutation in *Salmonella*.

Aromatic amines including aniline and *o*-toluidine are present in cigarette smoke condensate and some kinds of vegetables [6,7]. In addition, norharman and harman have been found in cigarette smoke condensate and cooked foods [8,9]. Thus, it is very likely that humans are simultaneously exposed to β-carbolines and aromatic amines. To clarify the significance of compounds like norharman and harman in the presence of aromatic amines to humans, much detailed data on levels of these compounds in the environment are required. In the present study, we therefore estimated the amounts of norharman and harman in mainstream and sidestream smoke of various brands of cigarettes, and also in various cooked foods.

2. Materials and methods

2.1. Materials

Six Japanese brands of cigarettes (non-filter, brand I, and five filter-tipped, brands II–IV) were smoked in an automatic smoking machine under standard conditions as described previously [10]. Samples of mainstream and sidestream cigarette smoke from 10 cigarettes in each case were collected on quartz-fiber filters.

Meats used in the present study were purchased at a local market and cooked in a routine fashion. In the case of broiled beef, chicken and mutton, slices were broiled for about 3 min on each side over a naked gas flame. Other cooked food samples were prepared as described previously [11,12]. Food-grade beef extract, used for preparation of soups, stews and gravy, was also obtained at a local market in Tokyo.

2.2. Quantification of norharman and harman in cigarette smoke condensates

The condensates of mainstream and sidestream smoke were extracted with 50 ml of methanol–chloroform (1:1 vol./vol.) three times, separately. Each extract was evaporated to dryness and the smoke condensate, dissolved in 20 ml of methanol, was diluted with 500 ml of water, and treated with 1 g of blue cotton (Funakoshi Pharmaceutical Co., Tokyo) three times, as previously reported [13]. The adsorbed material was extracted with methanol–ammonia solution, the eluate was evaporated and the residue, dissolved in methanol, was further purified with a cation exchange TIN-100 H05E fiber column

Table 1
Amounts of norharman and harman in mainstream and sidestream cigarette smoke condensates

Brand	Smoke condensate	Amount (ng/cigarette)	
	(mg/cigarette)	Norharman	Harman
Mainstream			
I	27.2	4240	2240
II	17.4	3210	1500
III	16.7	3080	1520
IV	12.9	1900	1050
V	3.0	900	360
VI	2.3	1470	670
Sidestream			
I	22.7	4760	2790
II	18.6	4130	2300
III	14.8	4410	2630
IV	12.0	4360	2100
V	16.8	8460	2320
VI	14.5	8990	3000

Table 2
Amounts of norharman and harman in cooked foods

Sample	Amount (ng/g cooked food)	
	Norharman	Harman
Grilled hamburger	5.98	3.63
Grilled beef steak	7.34	5.39
Pan-fried hamburger	12.0	7.00
Pan-fried steak	12.5	3.80
Pan-fried bacon	40.2	5.50
Pan-fried pork chop	2.39	0.62
Oven-broiled bacon	59.6	32.5
Oven-broiled roast beef	5.0	4.03
Oven-broiled pork chop	17.3	11.2
Broiled beef	795	169
Broiled chicken	622	133
Broiled mutton	458	67.7
Beef extract	93.8	377

(1.5 × 7 cm) and HPLC on a semi-preparative ODS column as described previously [13,14]. The fractions corresponding to the retention times of authentic norharman and harman were collected, and levels of the compounds were determined by HPLC using a combination of two analytical columns, a cation exchange TSKgel SP-2SW column (Tosoh Corp., Tokyo) and a YMC A303 column (Yamamura Chemical Laboratories, Kyoto), under the same conditions previously reported [14]. Norharman and harman were detected by their fluorescence with excitation and emission wavelengths of 260 nm and 430 nm, respectively.

UV and fluorescence emission spectra of norharman and harman in the samples were analyzed with an SPD-M6A photodiode array detector (Shimadzu Corp., Kyoto) and an FS-8011 fluorometric detector (Tosoh Corp.), respectively.

2.3. Quantification of norharman and harman in cooked foods

Samples of 5 g of cooked meat were homogenized in 50 ml of 0.1 N HCl three times, and the extracts were mixed with trichloroacetic acid and centrifuged to remove protein. The supernatant was neutralized with aqueous alkaline solution. In the case of a food-grade beef extract, a sample of 5 g was dissolved in 150 ml of water. These solutions were purified by blue cotton treatment, cation exchange fiber column chromatography and HPLC on a semi-preparative ODS column, then amounts of β-carbolines, norharman and harman, were analyzed by HPLC on SP-2SW and ODS columns under the same conditions used for the samples of cigarette smoke as described above.

2.4. Recoveries of norharman and harman

The recoveries of norharman and harman during the purification process were estimated by spiking with equivalent levels of authentic β-carbolines to those detected in samples of cigarette smoke and cooked food.

3. Results and discussion

Norharman and harman were detected in mainstream and sidestream smoke condensates of all the samples. The recoveries of norharman and harman during the purification process were 67.5 and 63.8%, respectively. In addition, the compounds detected by HPLC were confirmed to be norharman and harman by their UV and fluorescence emission spectra.

By correcting the amounts of these β-carbolines, norharman and harman, detected by HPLC for their recoveries, the levels in mainstream and sidestream smoke condensates were calculated (see Table 1). The values for norharman and harman in mainstream cigarette smoke condensates were 900–4240 ng and 360–2240 ng per cigarette, respectively. The levels of these β-carbolines in sidestream cigarette smoke condensates were 4130–8990 ng for norharman and 2100–3000 ng for harman, per cigarette, and thus considerably higher than those in mainstream cigarette smoke condensates. The levels of β-carbolines in mainstream and sidestream cigarette smoke condensates were not well correlated with the amounts of smoke condensates, as shown in Table 1.

Norharman and harman were also found to be present in all the samples of cooked foods. The amounts of these β-carbolines detected by HPLC were corrected for their recoveries during the purification process, estimated at 63.3% for norharman and 60.4% for harman, and the corrected levels are given in Table 2. Values for norharman and harman were 2.39–795 ng and 0.62–377 ng per g of cooked food, respectively. Among 13 cooked food samples,

norharman was most abundantly detected in broiled beef and harman in beef extract.

In the present study, all the samples of mainstream and sidestream cigarette smoke condensates contained norharman and harman. In addition to β-carbolines, mutagenic, carcinogenic α- and γ-carbolines have been shown to be formed by heating tryptophan [15,16]. Examples such as 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-9*H*-pyrido [2,3-*b*]indole (AaC) and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAaC), are present in mainstream smoke condensates at levels of 0.02–13.5 ng per cigarette and in sidestream smoke condensate at levels of 0.14–2.72 ng per cigarette [17]. These levels are much lower than those determined here for β-carbolines. Thus, β-carbolines appear to be produced by heating tryptophan in cigarette leaves at higher yields than α- and γ-carbolines.

Like cigarette smoke condensates, all the cooked food samples, such as grilled, pan-fried, oven-broiled and broiled meats and beef extract, were also found to contain the two β-carbolines, norharman and harman. Variations of the levels of the β-carbolines in cooked foods are probably due to differences in cooking conditions including temperature, heating time and water content, as well as the included tryptophan. Amounts of mutagenic and carcinogenic heterocyclic amines (HCAs) have been estimated in various cooked foods [18]. Among HCAs detected, PhIP was the most abundant, being present at levels of 0.56–69.2 ng/g. Values for other HCAs were 0.03–6.44 ng/g. Thus, the levels of norharman and harman are also much larger than those of HCAs in cooked foods.

The present study confirmed that the co-mutagens norharman and harman are widely distributed in our environment. Moreover, norharman and harman have been detected in all urine samples from healthy volunteers eating an ordinary diet, as well as from patients receiving parenteral alimentation [14]. From these observations, we can conclude that humans are continuously exposed to these β-carbolines, derived from endogenous and exogenous sources. It is now very important to study whether aminophenylnorharman is formed from norharman and aniline endogenously, and determine its biological activity, including carcinogenicity.

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References

[1] M. Nagao, T. Yahagi, M. Honda, Y. Seino, T. Matsushima, T. Sugimura, Demonstration of mutagenicity of aniline and o-toluidine by norharman, Proc. Jpn. Acad. 53B (1977) 34–37.

[2] M. Nagao, T. Yahagi, T. Sugimura, Differences in effects of norharman with various classes of chemical mutagens and amounts of S-9, Biochem. Biophys. Res. Commun. 83 (1978) 373–378.

[3] T. Sugimura, M. Nagao, K. Wakabayashi, Metabolic aspects of the comutagenic action of norharman. In: R. Snyder, D.J. Jollow, D.V. Parke, C.G. Gibson, J.J. Kocsis, C.M. Witmer (Eds.), Biological Reactive Intermediates-II, Chemical Mechanisms and Biological Effects Part B, Plenum Press, New York, 1982, pp. 1011–1025.

[4] M. Mori, Y. Totsuka, K. Fukutome, T. Yoshida, T. Sugimura, K. Wakabayashi, Formation of DNA adducts by the co-mutagen norharman with aromatic amines, Carcinogenesis 17 (1996) 1499–1503.

[5] Y. Totsuka, N. Hada, K. Matsumoto, N. Kawahara, Y. Murakami, Y. Yokoyama, T. Sugimura, K. Wakabayashi, Structural determination of a mutagenic aminophenylnorharman produced by the co-mutagen norharman with aniline, Carcinogenesis 19 (1998) 1995–2000.

[6] Int. Agency Res., Cancer, IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, Vol. 27, Aniline and Aniline Hydrochloride, Int. Agency Res Cancer, Lyon, 1982, pp. 39–61.

[7] Int. Agency Res., Cancer, IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans; Vol. 27, Ortho-Toluidine and Ortho-Toluidine Hydrochloride, Int. Agency Res. Cancer, Lyon, 1982, pp. 155–175.

[8] G.A. Gross, R.J. Turesky, L.B. Fay, W.G. Stillwell, P.L. Skipper, S.R. Tannenbaum, Heterocyclic aromatic amine formation in grilled bacon, beef and fish and in grill scrapings, Carcinogenesis 14 (1993) 2313–2318.

[9] E.H. Poindexter Jr, R.O. Carpenter, The isolation of harmane and norharmane from tobacco and cigarette smoke, Phytochemistry 1 (1962) 215–221.

[10] S. Sato, Y. Seino, T. Ohka, T. Yahagi, M. Nagao, T. Matsushima, T. Sugimura, Mutagenicity of smoke condensates from cigarettes, cigars and pipe tobacco, Cancer Lett. 3 (1977) 1–8.

[11] R. Sinha, N. Rothman, C.P. Salmon, M.G. Knize, E.D. Brown, C.A. Swanson, D. Rhodes, S. Rossi, J.S. Felton, O.A. Levander, Heterocyclic amine content in beef cooked by different methods to varying degrees of doneness and gravy made from meat drippings, Food Chem. Toxicol. 36 (1998) 279–287.

[12] R. Sinha, M.G. Knize, C.P. Salmon, E.D. Brown, D. Rhodes, J.S. Felton, O.A. Levander, N. Rothman, Heterocyclic amine content of pork products cooked by different methods and to varying degrees of doneness, Food Chem. Toxicol. 36 (1998) 289–297.

[13] H. Ushiyama, K. Wakabayashi, M. Hirose, H. Itoh, T. Sugimura, M. Nagao, Presence of carcinogenic heterocyclic amines in urine of healthy volunteers eating normal diet, but not of inpatients receiving parenteral alimentation, Carcinogenesis 12 (1991) 1417–1422.

[14] H. Ushiyama, A. Oguri, Y. Totsuka, H. Itoh, T. Sugimura, K. Wakabayashi, Norharman and harman in human urine, Proc. Jpn. Acad. 71B (1995) 57–60.

[15] T. Sugimura, T. Kawachi, M. Nagao, T. Yahagi, Y. Seino, T. Okamoto, K. Shudo, T. Kosuge, K. Tsuji, K. Wakabayashi, Y. Iitaka, Mutagenic principle(s) in tryptophan and phenylalanine pyrolysis products, Proc. Jpn. Acad. 53 (1977) 58–61.

[16] D. Yoshida, T. Matsumoto, Isolation of 2-amino-9*H*-pyrido[2,3-*b*]indole and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole as mutagens from pyrolysis product of tryptophan, Agric. Biol. Chem. 43 (1979) 1155–1156.

[17] K. Wakabayashi, I.S. Kim, R. Kurosaka, Z. Yamaizumi, H. Ushiyama, M. Takashasi, S. Koyota, A. Tada, H. Nukaya, S. Goto, T. Sugimura, M. Nagao, Identification of new mutagenic heterocyclic amines. In: R.H. Adamson, J.A. Custafsson, N. Ito, M. Nagao, T. Sugimura, K. Wakabayashi, Y. Yamazoe (Eds.), Heterocyclic Amines in Cooked Foods, Possible Human Carcinogens, Princeton Scientific Publishing Co., Princeton, NJ, 1995, pp. 39–49.

[18] K. Wakabayashi, M. Nagao, H. Esumi, T. Sugimura, Food-derived mutagens and carcinogens, Cancer Res. 52 (1992) 2092s–2098s.